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13. ABSTRACT (Maximum 200 Words) Toward addressing the functions of ER α in breast cancer, we have proposed to conditionally ablate ER- α gene in the mammary gland. A gene-targeting vector was generated from a 9.2 KB BamHI fragment containing the ER α exon 3. The construct was confirmed by restriction enzyme digestion and full length DNA sequencing. The linearized targeting vector was electroporated into mouse 129/J1 embryonic stem cells (ES) and G418 neomycin-resistant clones were expanded. Approximately 290 G418-resistant ES clones have been screened for the targeted allele in the mouse genome. Three targeted clones were identified with the 5' and 3' PCR. Southern Blot confirmed two targeted clones. The ES cells harboring the floxed ER α gene have been transfected with an expression vector carrying cre recombinase to delete the Neo gene from the mouse genome of the ES cells. After appropriate selection and characterization, these ES cells will be injected into blastocysts and returned to a pseudo-pregnant host of the same strain for generating the floxed ER- α mice. Crossing the homozygous floxed ER α mice with WAP-cre mice is expected to conditionally knockout ER α in lactating mice, allowing us to address many fundamental questions related to estrogen receptor and breast cancer.				
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INTRODUCTION

Breast cancer is among the most devastating diseases affecting women. The risk for a North American women getting breast cancer has doubled since 1940, and at present one woman in eight is at risk of developing the disease. It is generally believed that estrogens play a significant role in the development of breast cancer. The mitogenic action of estrogen is amplified by the hormone-dependent transcription factor, estrogen receptor (ER). Because of its pivotal role in the normal physiology of breast, ER has become a target for pharmacological intervention in breast cancer. This contention is strengthened by observations that the estrogen receptor is a hormone dependent transcription factor that regulates the expression of growth factors and protooncogenes in breast tumor cell lines. Moreover, the growth and progression of many breast cancers are dependent upon estrogen, making measurement of ER- α standard in the treatment decisions for patients with breast cancer. The ER-positive breast tumors are generally associated with faster growing and more aggressive tumors than the ER-negative tumors, which can be controlled with antiestrogen therapy. Towards understanding the role of ER in breast tumorigenesis, we have initiated a program, using transgenic mouse technology to ablate ER- α gene in the mammary gland. This mouse model is expected to be of great use in addressing the role of estrogen receptor in mammary tumorigenesis.

In the previous Progress Report we described our work with ER α mutant (K303R); yeast two-hybrid studies with ER α /ER β heterodimerization and non-genomic action of estradiol. In the present report, we will only present the progress summary. The current Progress Report will focus on our accomplishments related to the main focus of the DOD-funded project, i.e. generation of conditional ER α knock out (KO) mice.

BODY

1. Study on human ER-K303R mutant:

We have used the cDNA encoding ER (K303R) to stably transfect an ER null cell line called C4-12, using the G418 selection system. The stable cell line was tested for expression of the transgene by Western blot analysis.

2 Protein-protein interactions between ER α and ER β .

We have expanded the yeast two-hybrid experiments to study the interaction between ERs and the receptor coactivators and concluded that the F-domain of the receptor affects the estrogen-dependent interactions between p160 coactivators and the estrogen – bound ERs.

3. Studies with C4-12 breast cancer cells.

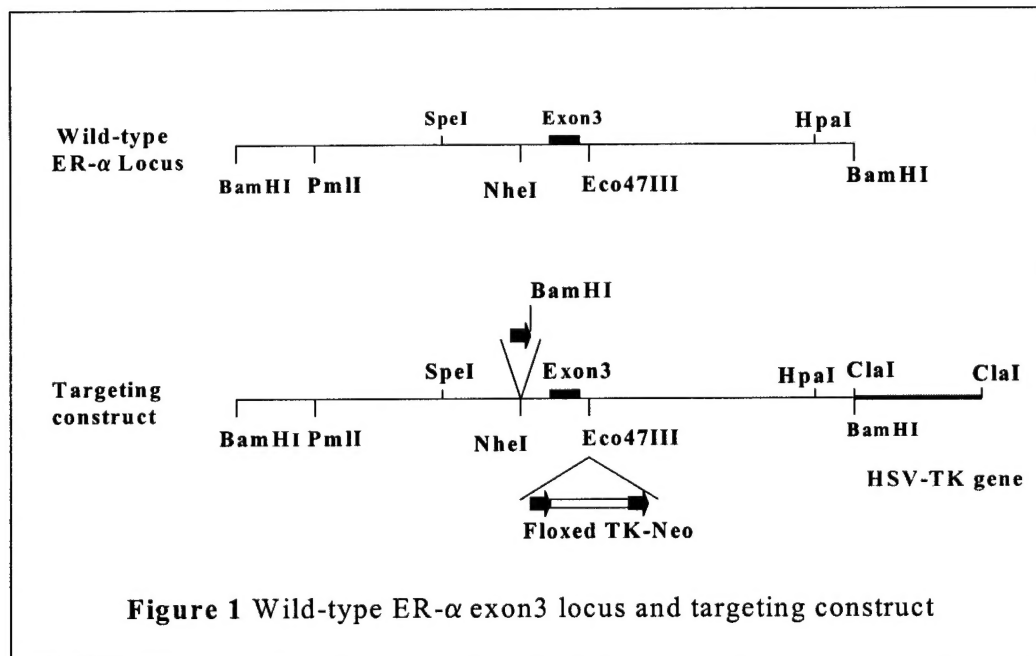
In this study, our most exciting achievement has been the establishment of breast cancer cell lines expressing ER α , ER β , K303R or ER α/β . We have now systematically begun to analyze these cell lines to determine the target genes for ERs. In the first set of such an experiments, we challenged the ER α expressing

cell line with vehicle, estradiol or an environmental estrogen bis phenol-A (BPA) and analyzed the gene expression pattern with microarray technology. As presented in Table-1, an interesting expression pattern of the target genes emerged. The intriguing observation is that some genes that are induced by estradiol are actually inhibited by BPA and the two ligands showed a different expression patterns. We have further confirmed the expression of some of these genes by real time PCR and focused on one of the protooncogenes, c-myb whose expression has been previously seen in breast cancer patients. We have cloned and sequenced its promoter, which revealed no consensus estrogen response element (ERE). It is likely that c-myb is induced by estradiol and BPA through AP-1 promoter elements.

5. Estrogen receptor- α gene-targeting vector and ES cell lines:

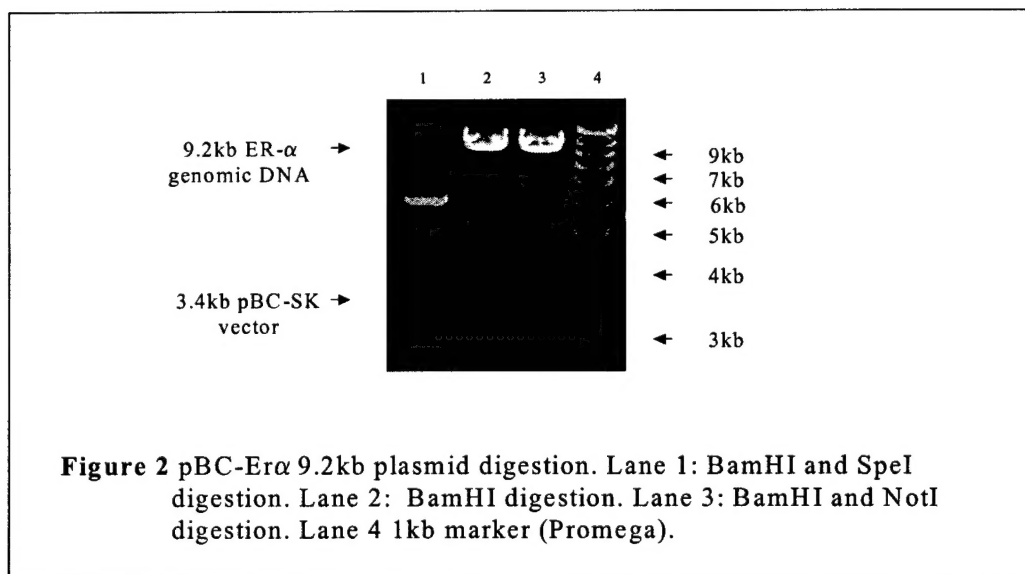
We have finished the construction of the ER α targeting vector and produced ES cell lines. The details are provided below:

Estrogen Receptor- α gene targeting vector: Construction & characterization.



In order to make the targeting vector, we originally focused to delete exon 2 and obtained a mouse genomic DNA clone harboring a 10kb BamHI fragment of mouse ER α . We however revisited the issue and decided to focus on deleting exon 3. This was prompted by the fact that knockout mice carrying exon 2 deletion in the genomic KO (reported by Ken Korach) some low levels of truncated ER α expression persisted.

We obtained the exon 3 DNA fragment (pBC-ER α -BamHI) from Jan Ake Gustafsson (Karolinska Institute). The pBC-ER α -BamHI construct was amplified and analyzed by restriction enzyme digestion and sequencing (fig. 1). We have confirmed that the 9.2 kb insert is the ER α genomic fragment and harbors exon3 (fig. 2).



Utilizing a series of cloning steps, we designed the ER α conditional Knockout-targeting construct according to a scheme presented in Figure1. In order to screen the positive ES cell clones more efficiently, we introduced a negative selection gene, HSV TK, into the ClaI site of pBCSK vector (Stratagene). The "Floxed TK-Neo cassette" was inserted into the Eco43III site on the 3' side of exon3. The 46bp LoxP-BamHI fragment was inserted into the NheI site on the 5' side of exon3. The targeting construct was analyzed for its accuracy by restriction enzyme analysis and DNA sequencing (fig 3). The complete sequence of the 12.6 kb "targeting construct" was sequenced from 5' and 3' ends of the genomic DNA insert. We then compared this DNA sequence with the Celera gene bank using the BLAST software and found 97% sequence homology with the mouse genome. At this stage we decided to electroporate the targeting construct into the ES cells

Electroporation of the targeting construct into ES cells.

The KO construct was linearized with PmlI and electroporated into embryonic stem (ES) cells. Most ES cells were killed by G418 treatment, but 30 single clones were obtained after the selection with G418 at a concentration of 240 μ g/ml. The ES clones were cultured in 24-well plates to full confluence and lysed overnight for the isolation of their genomic DNA. 5' and 3' PCR primers were designed to detect the targeted allele (fig. 4). The ES clones have been

screened with ERKO 3' and 5' primers to detect the 5.2kb and 4.7 kb targeted alleles, but our PCR screening did not find targeted clones. The reason that most ES cells were killed was because of the reduced G418 resistance, resulting from a natural mutation in the Neo gene on our construct.

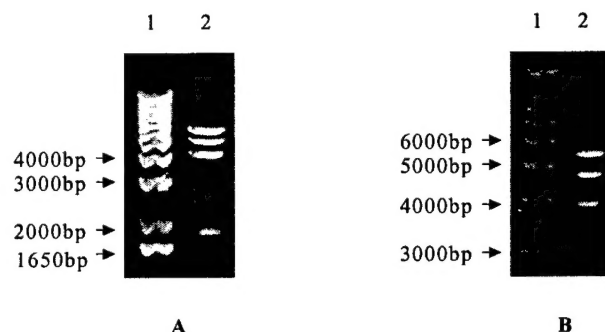


Figure 3 pBC-ER α KO construct BamHI digestion. Two BamHI sites have been introduced into the KO construct at LoxP-BamHI site and the 3' end of Floxed TK-Neo cassette. 4 bands are generated by BamHI (1.9kb, 3.9kb, 4.7kb and 5.4kb). Panel B is a longer run of Panel A. Lane 1 is Promega 1kb marker. Lane 2 shows KO construct BamHI digestion.

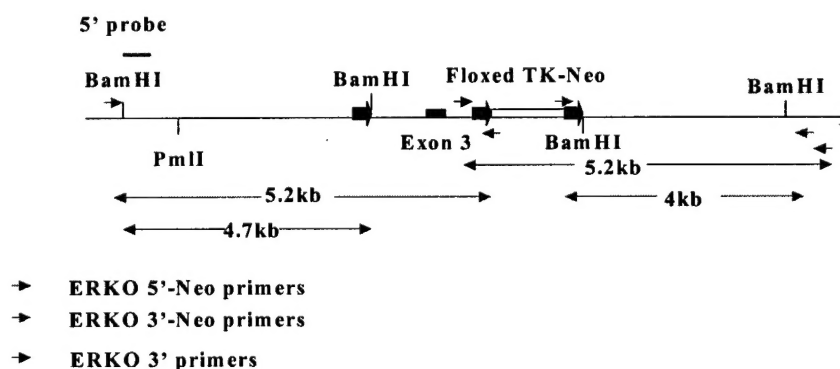


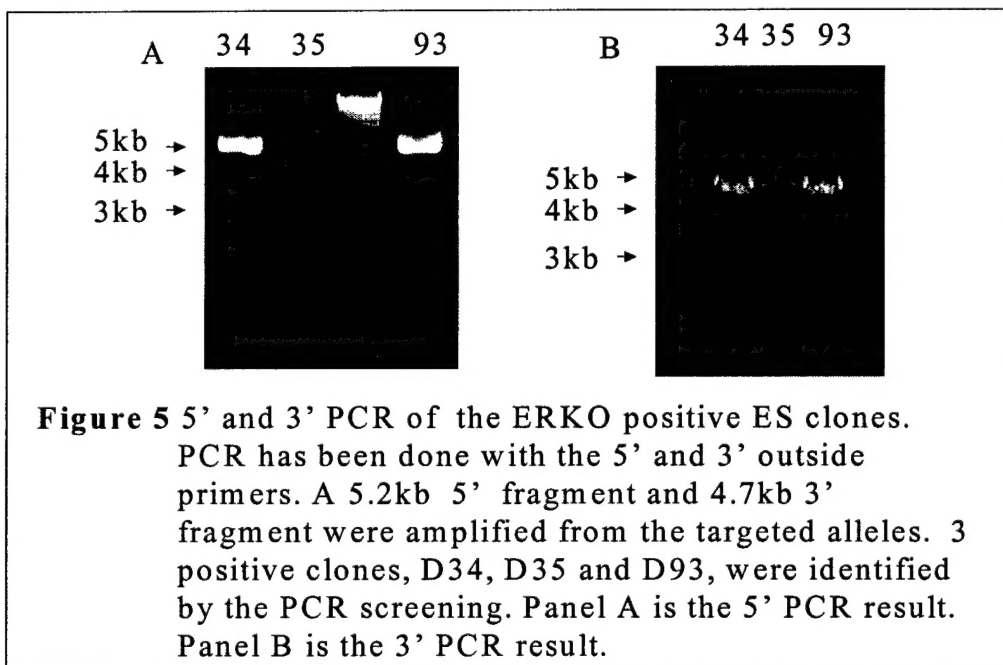
Figure 4 Strategies to screen and confirm the targeted allele. The ES clones will be screened with ERKO 3' primers for 5.2kb targeted allele. The targeted allele will be confirmed with ERKO 3'-Neo primers, ERKO 5'-Neo primers and Southern blot with 5' probe.

Repairing the Neo gene on the targeting construct

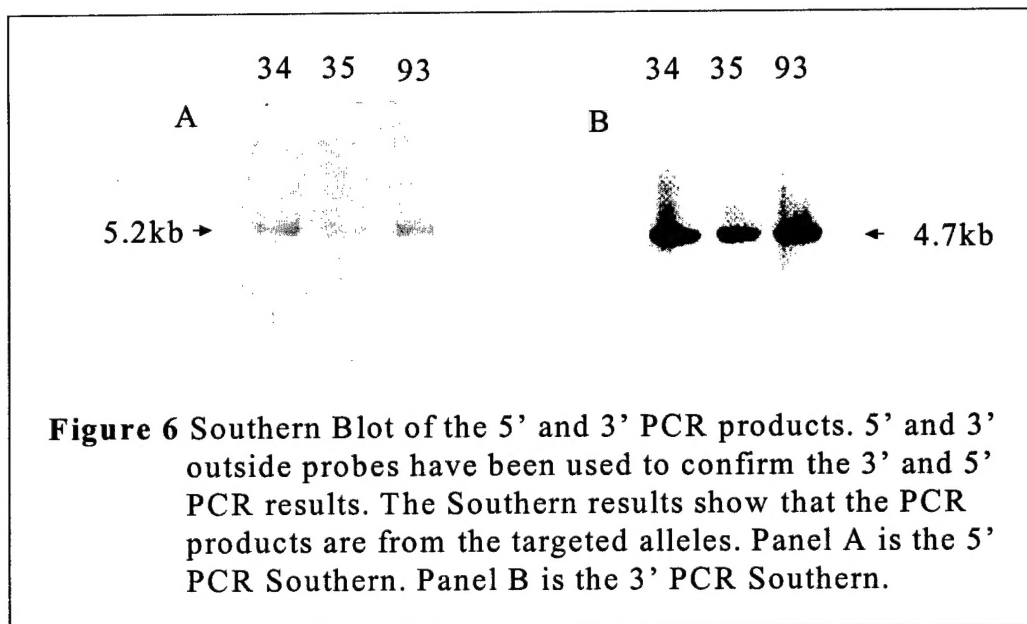
We decided to fix the mutation in our knockout construct with site-directed mutagenesis. The mutation was repaired on the pFloxNeo plasmid. The mutant Neo on the KO construct was then replaced with the fixed Neo gene. The KO construct was then linearized with PmlI and electroporated into ES cells again.

Screening of targeted ES clones

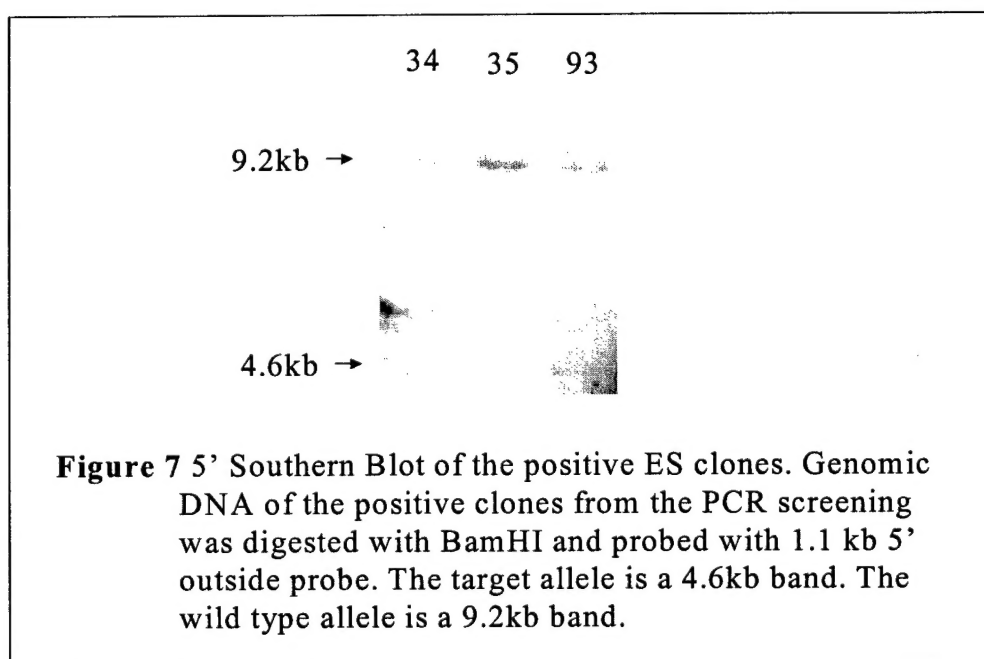
About 290 ES clones were amplified after the G418 selection. Genomic DNA was extracted from the lysate of the ES cells. 5' and 3' PCR from the flanking region of target to Neo gene was used to screen the targeted alleles. 3 positive clones were identified by PCR (figure 5).



For confirmation, the 5' and 3' PCR products of the positive clones were subjected to Southern Blot with 3' and 5' outside probes. The PCR Southern experiment confirmed that the PCR products are from the targeted alleles (figure 6).



Genomic DNA from the positive clones was digested with BamHI and probed with 5' outside probe, a 1.1 kb DNA fragment generated with NotI and PmlI double digestion on the ERKO construct. Two positive clones, D34 and D93, were confirmed by the Southern Blot (figure 7).



Cre recombinase treatment to delete Neo gene

The Cre recombinase has been electroporated into the mixed positive clones (D34 and D93). G418 has been added to the cells to select the G418 sensitive clones. The genomic DNA of the G418 sensitive clones will be screened with 5' and 3' outside primers. Since the cells are sensitive to G418, we do not expect to amplify DNA from clones that lose the Neo gene. These negative clones will be further screened with inside primers that amplify the region between LoxP1 and LoxP3. 700bp fragment will be amplified only if the Neo gene is deleted. A smaller DNA fragment will be amplified if the exon 3 is also deleted by Cre recombinase. The correct clones will be confirmed by Southern blot with 3' probe.

Proposed Changes for the Future Work:

We are not proposing any changes in the experimental plan. After confirming the integration of the targeting construct into the ES cells, they will be used to generate the mice carrying Floxed ER α gene. Once the colony is established, they will be crossed with transgenic mice carrying Cre recombinase gene under the whey acidic protein (WAP) promoter to obtain ER α conditional KO mice.

Key Research Accomplishments:

- Establishment of breast cancer cell lines expressing different ERs.
- Identification of the target genes of estrogen and BPA in breast cancer cell line expressing only ER α .
- Contribution the F-domain of ER α in the estrogen-dependent recruitment of coactivators.
- Construction of the ER α targeting vector and generation of ES cell lines for generating conditional ER α KO mice.

REPORTABLE OUTCOMES

Singleton, DW, Feng, Y. Burd, CJ and Khan, SA, "Non-genomic activity and subsequent c-fos induction by estrogen receptor ligands are not sufficient to promote DNA synthesis in human endometrial adenocarcinoma cells", *ENDOCRINOLOGY* 144: 121 - 128, 2003.

Singleton, D. and Khan, S, "Xenoestrogen exposure and mechanisms of endocrine disruption". *Front Biosci* ;8:110-8 (2003)

Singleton, D., and Khan, S. "Estrogen receptor dependence of MAP-kinase activation in breast and uterine tumor cells Annual Meeting of the "ENDOCRINE SOCIETY", Denver, Co (2001)

Singleton DW, Feng Y, Burd C, Khan SA. Nongenomic Action of Estrogen Receptor Ligands Is not Sufficient to Induce Proliferation of Human Endometrial Adenocarcinoma Cells. The Endocrine Society Meeting, June 2002

Cell Lines:

We have generated yeast cell lines transformed with different pairs of the estrogen receptor.

CONCLUSIONS

Estrogen receptor plays a key role in breast cancer. However, little is known about the involvement of the receptor in the progression of breast tumorigenesis. Towards achieving our overall goal of generating ER knockout mice for studying mammary tumorigenesis, we had proposed to generate ER- α conditional knockout mice. We have successfully generated the targeting construct for use in generating ER α knock-out mice. The work is progressing well.

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